

Specifically Targeting the CD22 Receptor of Human B-cell Lymphomas With RNA Damaging Agents: A New Generation of Therapeutics

MIRIAM HURSEY^a, DIANNE L. NEWTON^b, HANS J. HANSEN^c, DALE RUBY^b, DAVID M. GOLDENBERG^d and SUSANNA M. RYBAK^{a,*}

^aDevelopmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD 21702-1201, USA; ^bSAIC Frederick, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD 21702, USA; ^cImmunomedics Inc., Morris Plains, NJ 07950, USA; ^dGarden State Cancer Center, Belleville, NJ 07109, USA

(In final form 20 October 2001)

Targeting CD22 on human B-cells with a monoclonal antibody conjugated to a cytotoxic RNase causes potent and specific killing of the lymphoma cells *in vitro*. This translates to anti-tumor effects in human lymphoma models in SCID mice. RNA damage caused by RNases could be an important alternative to standard DNA-damaging chemotherapeutics. A second generation construct with an improved recombinant cytotoxic RNase is described. Targeted RNases may overcome problems of toxicity and immunogenicity associated with plant or bacterial toxin-containing immunoconjugates.

Keywords: Antibody; Ribonuclease; Cancer; Immunotoxin; Apoptosis; CD22

INTRODUCTION

Selective cytotoxicity using monoclonal antibodies is a major goal in drug development. Monoclonal antibody therapies have particularly improved for the treatment of non-Hodgkin's lymphomas (NHL). Encouraging response rates in patients treated with antibody constructs have been reported to range from 25 to 95% (reviewed in Ref. [1]). One of the best-characterized monoclonal antibody targets on B-cells is the CD22 antigen. It is attractive because it is not exposed on embryonic stem or pre-B cells nor is it normally shed from the surface of antigen-bearing cells [2]. A murine anti-CD22 monoclonal antibody (LL2, originally designated EPB-2 [3]) was developed for imaging and treatment of NHL. LL2 has a highly restricted specificity, yet is reactive with virtually all cases of NHL [4]. Five evaluable patients treated with radiolabeled murine, chimeric or humanized LL2 achieved treatment responses, including one complete response in a patient who received only "diagnostic" doses of [¹³¹I]-LL2 IgG [5]. Although these and other results ([1] and references therein) are promising, radiotherapy is toxic and the durability of the responses are not yet known. More recently humanized LL2, Epratuzumab, was reported to cause objective responses in NHL patients

with minimal toxicity when administered as a naked antibody [6].

Potency against cancer cells can be enhanced when the antibodies are attached to various types of effectors (e.g. drugs, toxins or radionuclides). Recently, an anti-CD22 antibody fused to a toxin variant was reported to be particularly effective in patients with hairy cell leukemia [7]. In the following pages, we explore the potent combination of anti-CD22-targeted RNA-damaging agents as possible therapeutics for the treatment of lymphoma. Certain RNA-damaging agents are human proteins, or closely related, and are already in clinical trials for cancer. These qualities as well as novel mechanisms may offer attractive options for antibody targeted therapies.

RNA AS A THERAPEUTIC TARGET

Nature has exploited the use of RNA as a molecular target because it is more structurally diverse than DNA. Like proteins, RNA can fold into complex tertiary structures that create multiple sites for molecular recognition. Furthermore, damaged RNA is not subject to repair mechanisms and is more accessible than nuclear DNA.

*Corresponding author. Tel: +1-301-846-5215. Fax: +1-301-846-7022. E-mail: rybak@ncifcrf.gov

Currently, there is interest in developing RNA-based therapeutics with RNA damaging enzymes, RNAses [8–10].

The appeal of RNA as a drug target has stimulated the investigation of possible therapeutic applications for more than 40 years. In 1955, bovine pancreatic RNase A injected into tumor-bearing mice was reported to impede tumor growth [11,12]. Thus, investigations into the clinical use of RNase A were initiated for the treatment of leukemia. Patients with chronic myelocytic leukemia were given daily s.c. injections of 0.5–1 mg of the bovine enzyme and were reported to have a decrease in spleen size and a general improvement [13].

Although RNase A did not enter the lexicon of anticancer drugs, an RNase is currently in clinical trials for a variety of cancers. Onconase (Alfacell Corporation, Bloomfield, NJ), an amphibian member of the pancreatic RNase A superfamily, was isolated from *Rana pipiens* oocytes by following cytotoxic activity against cancer cells *in vitro* [14–18] and *in vivo* [15]. It is the only unconjugated RNase currently in clinical trials. The efficacy of onconase can be increased in combination therapy with standard chemotherapeutic agents [16,17], even in the presence of the *mdr1* form of multidrug resistance [19]. Phase I and Phase I/II clinical trials of onconase as a single therapeutic agent in patients with a variety of solid tumors have recently been completed [20] and have progressed to Phase III clinical trials. Although onconase is an amphibian protein, there have been no problems associated with repeated administration in humans. This is most likely due, in part, to its positive charge and small size (12 kDa).

The potent cell killing activity of onconase is due to damage to tRNA [21,22] that causes a physiologically relevant death signal in mammalian cells [22]. Similar to DNA damage, the caspase cascade is activated, resulting in apoptosis. However, unlike DNA damage and apoptosis [23], onconase-induced apoptosis through tRNA damage is not affected by the absence of a functional p53 protein [22], which is yet another advantage of targeting RNA.

Although onconase is being evaluated as a single agent or in combination chemotherapy, its activity is markedly increased when linked to antibodies ([9] and references therein). To date, the most potent antibody-onconase combination is against the CD22 antigen; LL2 increases the lymphoma specific activity 10,000 fold [24].

RECOMBINANT CYTOTOXIC RNASES

The nature of the N-terminal is crucial to the cytotoxicity of RNases. Dimeric bovine seminal (BS) RNase expressed as a methionine (Met-1) fusion protein in the inclusion bodies of *E. coli* retained full enzymatic activity but expressed decreased cytotoxicity [25]. The X-ray crystallographic structure of onconase revealed that the N-terminal < Glu residue forms part of the active enzyme site [26]. All native cytotoxic amphibian RNases contain

an N-terminal < Glu amino acid residue. Recombinant amphibian RNases expressed as (Met-1) fusion proteins express neither catalytic nor cytotoxic activity [27–29]. Full activities are expressed when the < Glu is reconstituted [27,29,30] or modified to restore active site interactions [31].

CLONING A CYTOTOXIC RNASE WITH IMPROVED PROPERTIES

rapLR1 was cloned from a cDNA library using poly [A] RNA purified from *R. pipiens* liver [32]. An open reading frame (ORF) encoding a putative 127 amino acid protein was found at the 5' end of clone 5a1b starting from base 97. Amino acids 1–23 are characteristic of a signal peptide with a charged amino acid within the first five amino acids, a stretch of at least nine hydrophobic amino acids to span the membrane, and ending with a cysteine [33]. The putative signal peptide sequence is followed from amino acid 24–127 by a highly conserved but not identical amino acid sequence compared to onconase [34]. The four amino acid differences between the ORF of clone 5a1b and onconase include amino acid residues 11 Leu ↔ Ile, 20 Asn ↔ Asp, 85 Thr ↔ Lys and 103 His ↔ Ser, respectively. With the exception of the conservative change at amino acid residue 11, all the other amino acid conversions are between polar and charged amino acid residues. The changes in amino acid sequence cause substantial differences in both enzymatic and cytotoxic properties of expressed and purified *rapLR1* compared to onconase. Most notably, *rapLR1* is fully active without the N-terminal < Glu amino acid residue (IC_{50} s, 0.8 μ g/ml native onconase and *rapLR1*, respectively). This simplifies expression of recombinant protein since recombinant onconase without a modified N-terminal amino acid residue is at least 100 fold less active ($IC_{50} > 100 \mu$ g/ml, [27,28]).

TARGETING *rapLR1* TO LYMPHOMA CELLS WITH ANTI-CD22 ANTIBODIES

Intermolecular disulfide bond(s) between *rapLR1* and LL2 were formed by reacting *rapLR1* derivatized with the heterobifunctional cross-linking reagent, SPDP, with 2-iminothiolane-treated antibody. The resulting conjugate was separated from unreacted *rapLR1* by gel filtration on a TSK-3000 HPLC column. A molar excess of SPDP-*rapLR1* over the derivatized antibody yielded essentially all conjugate and no free antibody following a procedure used previously in the preparation of RNase conjugates [35–37]. The number of mols of *rapLR1* conjugated per mol antibody ranged between 2 and 3 mols *rapLR1*/mol LL2. The M_r of LL2-*rapLR1* on non-reducing SDS-PAGE was approximately 180 kDa, as expected for the monomeric antibody coupled to approximately 2.5 mols of *rapLR1*. No aggregates or degradation products were

TABLE I RNase activity of *rap*LR1 or LL2-*rap*LR1 conjugate

RNAse	pH*	Mols RNAse per mol Ab†	K_m μ M	K_{cat} s^{-1}	K_{cat}/K_m $M^{-1} s^{-1}$
<i>rap</i> LR1	6.0	NA‡	17.1	0.15	8800
LL2- <i>rap</i> LR1	6.0	2.6	17.1	0.08	4,700 (0.53)¶

* The RNase activity was measured at the pH most optimal for *rap*LR1. The data were derived from the initial rates of reactions containing 0.3 and 1.0 nM *rap*LR1 or 0.8 and 2.5 nM LL2-*rap*LR1 and varying (0.1–1 mg/ml) concentrations of the yeast tRNA substrate. The data for each enzyme concentration was pooled and averaged.

† The number of mols of RNAse conjugated per mol of antibody was determined spectrally at 412 nm by following the appearance of thionitrobenzoate ion (TNB). TNB is released from the 2-IT and DTNB-treated antibody as disulfide bonds between the RNAse and antibody are formed.

‡ NA, not applicable.

¶ The number in parentheses indicates the percentage activity compared with *rap*LR1.

detected on SDS-PAGE or HPLC analysis. The conjugate was tested for RNase activity and compared to the enzymatic activity of unconjugated *rap*LR1. While the K_m or affinity of conjugated *rap*LR1 for the substrate remained similar to that of *rap*LR1, the catalytic efficiency (K_{cat}/K_m) was found to decrease by 47% (Table I). All procedures used to link *rap*LR1–LL2 were derived from studies with LL2-onconase and details related to these were published recently [24].

Flow cytometry showed that the binding of LL2 to the CD22 antigen on human Daudi cells was the same for the native antibody or antibody conjugate (Fig. 1A). A rat monoclonal anti-idiotypic antibody, WN, to the LL2 antibody was previously developed as a surrogate antigen for determination of LL2 immunoreactivity [38]. Binding of LL2 to WN is not altered by conjugation to *rap*LR1 (Fig. 1B).

with human plasma at 37°C for up to three days without significant loss of activity (Fig. 2). Problems with aggregation of anti-CD22 toxin antibodies have been experienced [39]. Therefore, the stability of LL2-*rap*LR1 over time was assessed at different storage temperatures (Table II). Over 3.2 months, the percent of high M_r aggregates increased from 6.7% in the original conjugate to 13.1, 9.5 and 10.2% at temperatures of 4, –20 and –70°C, respectively. The change in biological activity (IC_{50}) over this time was not significantly affected (70 pM in the original conjugate and 90, 70 and 90 pM after 3.1 months at the above indicated temperatures). The results indicate that the LL2-*rap*LR1 is stable at physiological temperatures and can be stored without significant problems of aggregation.

LL2-*rap*LR1 FORMULATION CHARACTERISTICS

For therapeutic applications, a disulfide-linked chemical conjugate must be stable. LL2-*rap*LR1 was incubated

IN VITRO ACTIVITY OF LL2-*rap*LR1

LL2-*rap*LR1 was assayed for cytotoxicity by examining inhibition of protein synthesis on the Daudi human B-cell tumor cell line. Conjugation to the LL2 antibody resulted in an enhancement of *rap*LR1 specific cytotoxicity

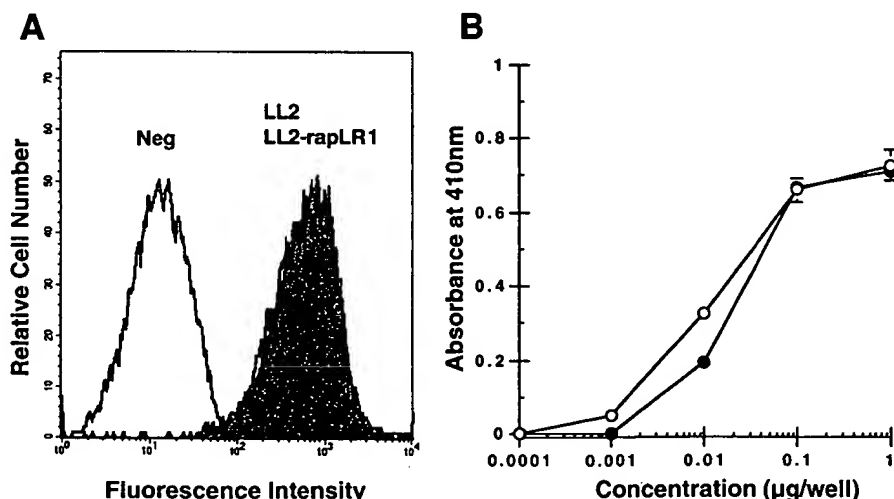


FIGURE 1 (A) Flow cytometric analysis of the binding of LL2 monoclonal antibody or the conjugate, LL2-*rap*LR1, to human Daudi lymphoma cells. Daudi lymphoma cells were incubated with 250 μ g/mL LL2 monoclonal antibody (LL2, heavy line) or LL2-*rap*LR1 (LL2-*rap*LR1, solid curve) and binding was detected with Alexa labeled antimouse antibody (1:200 dilution, Molecular Probes, Eugene, OR). As a negative control, target cells were incubated with Alexa labeled anti-mouse antibody alone (Neg). (B) Comparison of binding of LL2 and LL2-*rap*LR1 to WN IgG. Elisa plates were coated with 100 μ l of a 10 μ g/ml solution of WN IgG antibody. LL2 (open circles) or LL2-*rap*LR1 (solid circles), 100 μ l, at concentrations ranging from 0.001 to 10 μ g/ml were added to the plates. The binding was revealed with a peroxidase-conjugated antimouse immunoglobulin.

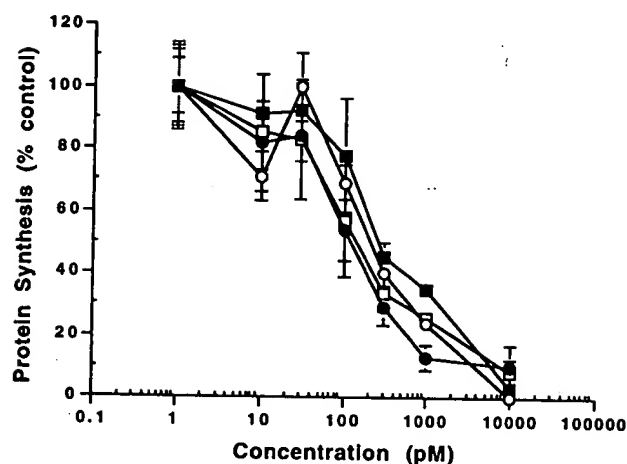


FIGURE 2 LL2-*rap*LR1 is stable in human plasma when incubated at 37°C over a three day period. LL2-*rap*LR1 was diluted 1:10 in pooled human plasma (Sigma, St Louis, MO) and incubated for 1, 2, and 3 days at 37°C. Cytotoxicity assays using Daudi lymphoma cells were performed to determine the activity of the treated conjugate in parallel with non-treated conjugate as described previously [24]. Day 0, solid circles; Day 1, open circles; Day 2, solid squares; Day 3, open squares.

by 25,000-fold (IC_{50} s, 0.06 and 1500 nM, *rap*LR1 and LL2-*rap*LR1, respectively).

The *in vitro* potency, as reflected by inhibition of protein synthesis, was enhanced by monensin, a carboxylic ionophore previously shown to enhance the activity of anti-CD22 immunotoxins (reviewed in Ref. [40]). monensin enhanced cytotoxicity 10-fold on Daudi cells (IC_{50} s, 200 and 20 pM in the absence and presence of monensin, respectively). Similarly monensin potentiated LL2-onconase activity [24], indicating that the routing of the CD22-*rap*LR1 conjugate is not altered. The increase in cytotoxicity was due to specifically binding the CD22 antigen. Competition with excess LL2 antibody (300 nM) completely abrogated the cytotoxicity of the conjugate. Moreover, LL2-*rap*LR1 did not inhibit protein synthesis in Jurkat T-cells that do not express the CD22 antigen, even though this cell line was more sensitive to unconjugated *rap*LR1 than the Daudi B-cell lymphoma cell line (*rap*LR1 IC_{50} , 200 and 1500 nM on Jurkat and Daudi cells, respectively).

IN VIVO EFFECTS OF LL2-*rap* LR1: NON-SPECIFIC TOXICITY IN MICE

To test non-specific toxic side effects, LL2-*rap*LR1 was injected (i.p.) four times a day for five days. All mice survived total doses of 300, 400 and 500 mg/kg, showing only a reversible weight loss upon cessation of treatment (Fig. 3). The highest dose of the conjugate tested (600 mg/kg) was similarly tolerated during administration, but resulted in drug related death 15 days after administration of the conjugate. Comparable anti-CD22 toxin conjugates are considerably more toxic to mice (LD_{50} s, 0.5–80 mg/kg) [41–46].

IN VIVO EFFECTS OF LL2-*rap* LR1: TREATMENT OF MINIMAL AND ADVANCED SYSTEMIC DAUDI LYMPHOMA IN SCID MICE

Intravenous injection (i.v.) of Daudi cells results in widely disseminated neoplasia with a more rapid onset of death compared to mice inoculated in the peritoneal cavity (i.p.) with the same number of tumor cells [47]. Compression of the spinal cord causing hind limb paresis was previously shown to be predictive of survival time, and was used as an end point (MPT) when Daudi cells were injected intravenously. Actual survival is the endpoint when tumor cells are injected into the peritoneal cavity (MST). Excellent increases in lifespan were reported previously in this model using the LL2-onconase conjugate [24], as well as with another anti-CD22-onconase conjugate (RFB4-onconase, S.M.R., unpublished results). In all of those treatment experiments the conjugate was always superior to the individual components or a mixture of the individual components.

In accord with the LL2-onconase studies, enhanced survival of mice after treatment with the LL2-*rap*LR1 conjugate is obtained (Table III). The MST (110 days) of mice treated with LL2-*rap*LR1 (i.p.) one day after tumor cell injection surpassed that of the other groups that included mice injected with PBS (MST, 55 days; $P = 0.0005$), or a mixture of unconjugated LL2 and

TABLE II Stability of LL2-*rap*LR1 stored at different temperatures*

Time (months)	Temperature (°C)	High MW aggregates (%)	Conjugate (%)	IC_{50} (pM)
0	4	6.7		
1.8	4	13.8	93.4	70
	-20	9.5	86.2	60
	-70	8.2	90.5	60
3.2	4	13.1	91.7	70
	-20	9.5	86.8	90
	-70	10.2	90.5	70
			90.0	90

*LL2-*rap*LR1 was aliquotted and stored at the temperatures indicated in the Table. At the noted time points, an aliquot (50 µg) was removed and analyzed by HPLC chromatography on a Toyo Soda TSK 3000 SW column (Toso Haas, Montgomeryville, PA) as described [24]. The integrated areas of peaks eluting between 19 and 29 min were added together (100%). High molecular weight aggregates (High MW aggregates) eluted between 19 and 22 min; the conjugate eluted between 23 and 29 min. Cytotoxicity assays were performed as described [24] against human Daudi B-cell lymphoma cells on the same aliquot as was analyzed by HPLC. The IC_{50} , the concentration of test sample that inhibits protein synthesis by 50%, was determined from semilogarithmic plots in which proteins synthesis as a percentage of control (buffer-treated cells) was plotted versus test protein concentration.

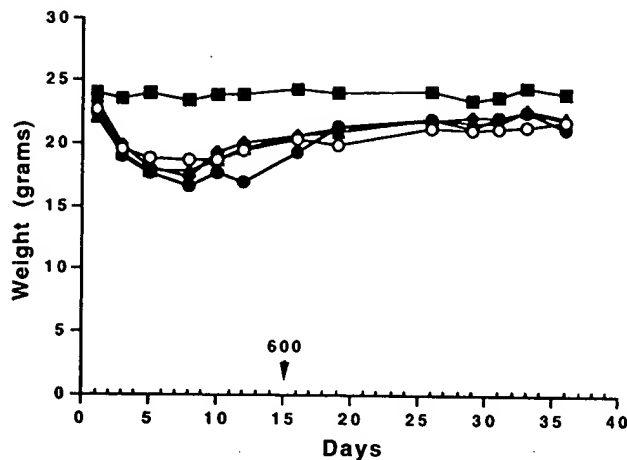


FIGURE 3 Toxicity in mice of LL2-rapLR1. Proteins diluted in phosphate buffered saline (PBS) were administered i.p. four times daily for five consecutive days to SCID mice. Mice were observed for signs of toxicity and weighed daily for several weeks after the last administration of drug. PBS, solid squares; 300 mg/kg LL2-rapLR1, open circles; 400 mg/kg LL2-rapLR1, solid triangles; 500 mg/kg LL2-rapLR1, solid diamonds; 600 mg/kg LL2-rapLR1, solid circles.

rapLR1 (MST, 76 days; $P = 0.0002$). Again, the covalently linked conjugate significantly increased the lifespan of Daudi-bearing mice over that of vehicle injected mice by 100%, when injected one day after tumor cell injection. The enhanced survival of the mixture of LL2 and rapLR1 is due to the antibody, and is consistent with the previously reported work [24] as well as responses of lymphoma patients to LL2 in human clinical trials [5]. The conjugate also was effective in treating mice with a more advanced tumor burden (Table III). In this model, tumor cells were injected i.v. and treatment was begun six days after tumor cell injection. The route of treatment was also examined in the advanced tumor burden model. Administration of conjugate intravenously was more effective (MPT 83 days; $p = 0.0002$) than intraperitoneal administration (MPT 62 days; $p = 0.04$), although both treatments increased survival (ILS, 102 and 51%, respectively).

TABLE III Treatment of minimal or advanced Daudi lymphoma disease with LL2-rapLR1 or component proteins*

Drug	Route of injection		MST/MPT†	% ILS‡
	Tumor Cells	Agent		
PBS	ip ¹	ip	55	
LL2+rapLR1	ip	ip	76	38
LL2-rapLR1	ip	ip	110	100
PBS	iv ¹	iv	41	
LL2+rapLR1	iv	iv	48	17
		ip	48	17
LL2-rapLR1	iv	iv	83	102
		ip	62	51

* Daudi lymphoma cells (5×10^6 cells) were injected either ip followed one day later for an additional four consecutive days or iv followed six days later for an additional four consecutive days with ip or iv injections of PBS, 80 μ g LL2+20 μ g rapLR1 or 100 μ g LL2-rapLR1.

† MST, median survival time; MPT, median paralysis time.

‡ % ILS, per cent increase in life span.

¹ ip, intraperitoneally; iv, intravenously.

In summary, we have found that three different formulations of anti-CD22 RNase (LL2-onconase; LL2-rapLR1 and RFB4-onconase) conjugates elicit potent effects against an aggressive model of disseminated murine lymphoma. All of the results of these treatment experiments were accomplished with a total dose of 25 mg/kg administered over five contiguous days. Preliminary experiments now indicate that doses as low as 0.25 mg/kg are as effective. Different schedules of administration are being examined to try to optimize treatment, since the effects are lost once the tumor cells escape to compartments not accessible to the conjugate (e.g. the spinal cord). This recombinant RNase conjugated to a CD22, B-cell antibody appears to hold promise as a new class of targeted therapeutics for B-cell malignancies.

Acknowledgements

The publisher or recipient acknowledges right of the U.S. Government to retain a nonexclusive, royalty-free license in and to any copyright covering the article. This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. NO1-CO-56000. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

References

- [1] Press, O.W. (1998) "Prospects for the management of non-Hodgkin's lymphomas with monoclonal antibodies and immunoconjugates", *Cancer Journal Scientific American* 4, S19-S26.
- [2] Li, J.L., Shen, G.L., Ghetie, M.A., May, R.D., Till, M., Ghetie, V., Uhr, J.W., Janossy, G., Thorpe, P.E., Amlot, P. and Vitetta, E.S. (1989) "The epitope specificity and tissue reactivity of four murine monoclonal anti-CD22 antibodies", *Cellular Immunology* 118, 85-99.
- [3] Pawlak-Byczkowska, E.J., Hansen, H.J., Dion, A.S. and Goldenberg, D.M. (1989) "Two new monoclonal antibodies, EPB-1 and EPB-2 reactive with human lymphoma", *Cancer Research* 49, 4568-4577.
- [4] Stein, R., Belisle, E., Hansen, H.J. and Goldenberg, D.M. (1993) "Epitope specificity of the anti-B-cell lymphoma monoclonal antibody, LL2", *Cancer Immunology and Immunotherapy* 37, 293-298.
- [5] Juweid, M., Sharkey, R.M., Markowitz, A., Behr, T., Swayne, L.C., Dunn, R., Hansen, H.J., Shevitz, J., Leung, S.O., Rubin, A.D. and Goldenberg, D.M. (1995) "Treatment of non-Hodgkin's lymphoma with radiolabeled murine, chimeric or humanized LL2, an anti-CD22 monoclonal antibody", *Cancer Research* 55, 5899s-5907s.
- [6] Leonard, J.P., Coleman, M., Schuster, M.W., Feldman, E.J., Chadburn, A., Ely, S., Sharkey, R.M., Hansen, H.J., Wegener, W.A. and Goldenberg, D.M. (2000) "CD-22 directed immunotherapy of NHL with Epratuzumab results in objective responses with minimal toxicity", *Cancer Biotherapy and Radiopharmaceuticals* 15, 104.
- [7] Kreitman, R.J., Wilson, W.H., Bereron, K., Raggio, M., Stetler-Stevenson, M., Fitzgerald, D.J. and Pastan, I. (2001) "Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy-resistant hairy-cell leukemia", *New England Journal of Medicine* 345, 241-247.

- [8] Schein, C.H. (1997) "From housekeeper to microsurgeon: the diagnostic and therapeutic potential of ribonucleases", *Nature Biotechnology* 15, 529–536.
- [9] Rybak, S.M. and Newton, D.L. (1999) "Natural and engineered cytotoxic ribonucleases: therapeutic potential", *Experimental Cell Research* 253, 325–335.
- [10] Leland, P.A. and Raines, R.T. (2001) "Cancer Chemotherapy—ribonucleases to the rescue", *Chemical Biology* 8, 405–413.
- [11] Ledoux, L. (1955) "Action of ribonuclease on two solid tumors *in vivo*", *Nature* 176, 36–37.
- [12] Ledoux, L. (1955) "Action of ribonuclease on certain ascites tumours", *Nature* 175, 258–259.
- [13] Aleksandrowicz, J. (1958) "Intracutaneous ribonuclease in chronic myelocytic leukemia", *Lancet* 2, 420.
- [14] Darzynkiewicz, Z., Carter, S.P., Mikulski, S.M., Ardelt, W.J. and Shogen, K. (1988) "Cytostatic and cytotoxic effects of Pannon (P-30 protein) a novel anti-cancer agent", *Cell Tissue Kinetics* 21, 169–182.
- [15] Mikulski, S.M., Ardelt, W., Shogen, K., Bernstein, E.H. and Menduke, H. (1990) "Striking increase of survival of mice bearing M109 Madison Carcinoma treated with a novel protein from amphibian embryos", *Journal of the National Cancer Institute* 82, 151–153.
- [16] Mikulski, S.M., Viera, A., Ardelt, W., Menduke, H. and Shogen, K. (1990) "Tamoxifen and trifluoroperazine (Stelazine) potentiate cytostatic/cytotoxic effects of P-30 protein, a novel protein possessing anti-tumor activity", *Cell Tissue Kinetics* 23, 237–246.
- [17] Mikulski, S.M., Viera, A., Darzynkiewicz, A. and Shogen, K. (1992) "Synergism between a novel amphibian oocyte ribonuclease and lovastatin in inducing cytostatic and cytotoxic effects in human lung and pancreatic carcinoma cell lines", *British Journal of Cancer* 66, 304–310.
- [18] Mikulski, S.M., Viera, A. and Shogen, K. (1992) "In vitro synergism between a novel amphibian oocyte ribonuclease (Onconase) and tamoxifen, lovastatin and cisplatin in human OVCAR-3 ovarian carcinoma cell line", *International Journal of Oncology* 1, 779–785.
- [19] Rybak, S.M., Pearson, J.W., Fogler, W.F., Volker, K., Spence, S.E., Newton, D.L., Mikulski, S.M., Ardelt, W., Riggs, C.W., Kung, H.F. and Longo, D.L. (1996) "Enhancement of vincristine cytotoxicity in drug-resistant cells by simultaneous treatment with Onconase, an antitumor ribonuclease", *Journal of the National Cancer Institute* 88, 747–753.
- [20] Mikulski, S.M., Grossman, A.M., Carter, P.W., Shogen, K. and Costanzi, J.J. (1993) "Phase I human clinical trial of ONCONASE (P-30 protein) administered intravenously on a weekly schedule in cancer patients with solid tumors", *International Journal of Oncology* 3, 57–64.
- [21] Lin, J.J., Newton, D.L., Mikulski, S.M., Kung, H.F., Youle, R.J. and Rybak, S.M. (1994) "Characterization of the mechanism of cellular and cell free protein synthesis inhibition by an anti-tumor ribonuclease", *Biochemical and Biophysical Research Communications* 204, 156–162.
- [22] Iordanov, M.S., Ryabinina, O.P., Wong, J., Dinh, T.-H., Newton, D.L., Rybak, S.M. and Magun, B.E. (2000) "Molecular determinants of programmed cell death induced by the cytotoxic ribonuclease onconase: evidence for cytotoxic mechanisms different from inhibition of protein synthesis", *Cancer Research* 60, 1983–1994.
- [23] Benhattar, J., Cerottini, J.P., Saraga, E., Methez, G. and Givel, J.C. (1996) "p53 mutations as a possible predictor of response to chemotherapy in metastatic colorectal carcinomas", *International Journal of Cancer* 69, 190–192.
- [24] Newton, D.L., Hansen, H.J., Mikulski, S.M., Goldenberg, D.M. and Rybak, S.M. (2001) "Potent and specific antitumor activity of an anti-CD22-targeted cytotoxic ribonuclease: potential for the treatment of non-Hodgkin lymphoma", *Blood* 97, 528–535.
- [25] Adinolfi, B.S., Cafaro, V., D'Alessio, G. and Di Donato, A. (1995) "Full antitumor action of recombinant seminal ribonuclease depends on the removal of its N-terminal methionine", *Biochemical and Biophysical Research Communications* 213, 525–532.
- [26] Mosimann, S.C., Ardelt, W. and James, M.N.G. (1994) "Refined 1.7 Å X-ray crystallographic structure of P-30 protein, an amphibian ribonuclease with anti-tumor activity", *Journal of Molecular Biology* 236, 1141–1153.
- [27] Boix, E., Wu, Y., Vasandani, V.M., Saxena, S.K., Ardelt, W., Ladner, J. and Youle, R.J. (1996) "Role of the N terminus in RNase A homologues: differences in catalytic activity, ribonuclease inhibitor interaction and cytotoxicity", *Journal of Molecular Biology* 257, 992–1007.
- [28] Newton, D.L., Xue, Y., Boque, L., Wlodawer, A., Kung, H.F. and Rybak, S.M. (1997) "Expression and characterization of a cytotoxic human–frog chimeric ribonuclease: potential for cancer therapy", *Protein Engineering* 10, 463–470.
- [29] Huang, H.-C., Wang, S.-C., Leu, Y.-J., Lu, S.-C. and Liao, Y.-D. (1998) "The *Rana catesbeiana* rer gene encoding a cytotoxic ribonuclease", *Journal of Biological Chemistry* 273, 6395–6401.
- [30] Notomista, E., Cafaro, V., Fusiello, R., Bracale, A., D'Alessio, G. and Donato, D. (1999) "Effective expression and purification of recombinant onconase, an antitumor protein", *FEBS Letters* 463, 211–215.
- [31] Newton, D.L., Boque, L., Wlodawer, A., Huang, C.Y. and Rybak, S.M. (1998) "Single amino acid substitutions at the N-terminus of a recombinant cytotoxic ribonuclease markedly influence biochemical and biological properties", *Biochemistry* 37, 5173–5183.
- [32] Chen, S.L., Le, S.Y., Newton, D.L., Maizel, Jr, J.V. and Rybak, S.M. (2000) "A gender specific mRNA encoding a cytotoxic ribonuclease contains a 3' UTR of unusual length and structure", *Nucleic Acids Research* 28, 2375–2382.
- [33] Vonheijne, G. (1983) "Patterns of amino acids near signal-sequence cleavage sites", *European Journal of Biochemistry* 133, 17–21.
- [34] Ardelt, W., Mikulski, S.M. and Shogen, K. (1991) "Amino acid sequence of an anti-tumor protein from *Rana pipiens* Oocytes and early embryos", *Journal of Biological Chemistry* 266, 245–251.
- [35] Rybak, S.M., Saxena, S.K., Ackerman, E.J. and Youle, R.J. (1991) "Cytotoxic potential of ribonuclease and ribonuclease hybrid proteins", *Journal of Biological Chemistry* 266, 21202–21207.
- [36] Rybak, S.M., Newton, D.L., Mikulski, S.M., Viera, A. and Youle, R.J. (1993) "Cytotoxic Onconase and ribonuclease A chimeras: comparison and *in vitro* characterization", *Drug Delivery* 1, 3–10.
- [37] Newton, D.L., Ilcail, O., Laske, D.W., Oldfield, E., Rybak, S.M. and Youle, R.J. (1992) "Cytotoxic ribonuclease chimeras: targeted tumoricidal activity *in vitro* and *in vivo*", *Journal of Biological Chemistry* 267, 19572–19578.
- [38] Losman, M.J., Leung, S.O., Shih, L.B., Shevitz, J., Shukla, R., Haraga, L., Goldenberg, D.M. and Hansen, H.J. (1995) "Development and evaluation of the specificity of a rat monoclonal anti-idiotypic antibody, WN, to an anti-B-cell lymphoma monoclonal antibody, LL2", *Cancer Research* 55, 5978s–5982s.
- [39] Messmann, R.A., Vitetta, E.S., Headlee, D., Senderowicz, A.M., Figg, W.D., Schindler, J., Michiel, D.F., Creekmore, S., Steinberg, S.M., Kohler, D., Jaffe, E.S., Stetler-Stevenson, M., Chen, H., Ghetie, V. and Sausville, E.A. (2000) "A phase I study of combination therapy with immunotoxins IgG-HD37-deglycosylated Ricin A chain (dgA) and IgG-RFB4-dgA (combotox) in patients with refractory CD19 (+), CD22 (–) B cell lymphoma", *Clinical Cancer Research* 6, 1302–1313.
- [40] Casellas, P. and Jansen, F.K. (1988) "Immunotoxin enhancers", In: Frankel, A.E., ed. *Immunotoxins* (Kluwer Academic Publishers, Boston, Dordrecht, Lancaster), pp 351–369.
- [41] Kreitman, R.J., Hansen, H.J., Jones, A.L., Fitzgerald, D.J.P., Goldenberg, D.M. and Pastan, I. (1993) "Pseudomonas Exotoxin-based immunotoxins containing the antibody LL2 or LL2-Fab' induce regression of subcutaneous human B-cell lymphoma in mice", *Cancer Research* 53, 819–825.
- [42] Mansfield, E., Pastan, I. and Fitzgerald, D.J. (1996) "Characterization of RFB4-Pseudomonas exotoxin A immunotoxins targeted to CD22 on B-cell malignancies", *Bioconjugate Chemistry* 7, 557–563.
- [43] Mansfield, E., Amlot, P., Pastan, I. and Fitzgerald, D. (1997) "Recombinant RFB4 immunotoxins exhibit potent cytotoxic activity for CD22-bearing cells and tumors", *Blood* (1997), 2020–2026.
- [44] Ghetie, M.A., Tucker, K., Richardson, J., Uhr, J.W. and Vitetta, E.S. (1992) "The anti-tumor activity of an anti-CD22 immunotoxin in SCID mice with disseminated Daudi lymphoma is enhanced by either an anti-CD19 antibody or an anti-CD19 immunotoxin", *Blood* 84, 702–707.
- [45] Van Hoesen, P., Preijers, F., Voosterhout, Y. and Witte, T.D. (1996) "Highly potent CD22-recombinant ricin A results in complete cure

- of disseminated malignant B-cell xenografts in SCID mice but fails to cure solid xenografts in nude mice", *International Journal of Cancer* **68**, 378–383.
- [46] Bolognesi, A., Tazzari, P., Olivieri, F., Polito, L., Lemoli, R., Terenzi, A., Pasqualucci, L., Falini, B. and Stirpe, E. (1998) "Evaluation of immunotoxins containing single-chain ribosome-inactivating proteins and an anti-CD22 monoclonal antibody (OM124): *in vitro* and *in vivo* studies", *British Journal of Haematology* **101**, 179–188.
- [47] Ghetie, M.A., Richardson, J., Tucker, T., Jones, D., Uhr, J.W. and Vitetta, E.S. (1990) "Disseminated or localized growth of a human B-cell tumor (Daudi) in SCID mice", *International Journal of Cancer* **45**, 481–485.